Identification of Mycotoxin-Producing Fungal Strains: A Step in the Isolation of Compounds Active against Rice Fungal Diseases

Boniface Okeke,† Françoise Seigle-Murandi,*,† Régine Steiman,† Jean-Louis Benoit-Guyod,‡ and Mourad Kaouadji§

Groupe pour l'Etude du Devenir des Xénobiotiques dans l'Environnement (GEDEXE), Laboratoire de Botanique, Cryptogamie, Biologie Cellulaire et Génétique, and Laboratoire de Toxicologie et Ecotoxicologie, UFR de Pharmacie, Université Joseph Fourier (Grenoble I), BP 138, 38243 Meylan Cédex, France, and Laboratoire de Pharmacognosie, UFR de Pharmacie, Université Joseph Fourier (Grenoble I), Domaine de la Merci, 38706 La Tronche Cédex, France

The simultaneous occurrence of very toxic mycotoxins with other useful fungal metabolites is a major obstacle in assays for new antifungal molecules. Screening of 67 fungal extracts in ethyl acetate to identify those with high contents of a known mycotoxin, patulin, has been undertaken. The extracts were earlier found to inhibit the *in vitro* growth on solid malt extract medium of three important rice pathogens: *Pyricularia oryzae*, *Drechslera oryzae*, and *Gerlachia oryzae*. *Eupenicillium javanicum*, *Penicillium cyaneum*, *Penicillium diversum*, *Penicillium duclauxii*, *Penicillium echinulatum*, *Penicillium glabrum*, *Penicillium lignorum*, *Alternaria tenuissima*, *Chaetomium atrobrunneum*, *Scopulariopsis flava*, and a *Stemphylium* sp. are reported for the first time as patulin producers. Pure patulin was observed to inhibit the growth of *D. oryzae* and *G. oryzae* at minimum concentrations of 0.50 and at 0.25 μg/mL, respectively, for *P. oryzae*. The diameter of the zones of inhibition increased with patulin concentration. There were low correlations between the quantity of patulin in the extracts and the level of *in vitro* inhibition of mycelial growth of each target phytopathogen.

INTRODUCTION

The simultaneous occurrence of highly toxic mycotoxins such as citrinin, ochratoxin A, patulin, and zearalenone with other beneficial molecules in extracts from natural sources such as micromycetes often represents a major obstacle in the isolation of vital antibiotic and antifungal (Steiman et al., 1989) as well as other agropharmacotherapeutic agents. It is therefore important to identify these mycotoxins.

Patulin is a mycotoxin that has been isolated from several micromycetes, especially from strains of *Aspergillus*, *Byssochlamys*, *Gymnoascus*, *Paecilomyces*, and *Penicillium* (Betina, 1989; Frisvad and Filtenborg, 1989). Among other micromycetes added recently to the list of patulin producers were some strains of *Mucor* and Deuteromycetes (Steiman et al., 1989). Several works have highlighted the danger represented by the presence of patulin in the environment (Lindroth and Wright, 1990; Paucod et al., 1990; Riley and Showker, 1991).

After a previous systematic assay of 1060 fungal metabolite extracts for inhibition of spore germination or the growth of mycelia of three important rice pathogens (Okeke et al., 1991)—*Pyricularia oryzae* Cavara [teleomorph *Magnaporthe grisea* (Hebert) Yaegashe and Udagawa], *Drechslera oryzae* (Breda de Haan) Subramanian and Jain = *Helminthosporium oryzae* Breda de Haan, and *Gerlachia oryzae* (Hashioka and Yokogi) W. Gams = *Rhynchosporium oryzae* Hashioka and Yokogi—the identification of the active extracts containing high levels of patulin was undertaken using a spectrophotometer at UV range and thin-layer chromatography (TLC). It was confirmed and quantified by high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) data.

Apart from identifying active fungal extracts with high patulin contents, this work has added new strains of fungi to the increasing list of patulin-producing micromycetes. This may be important in research works concerning the quality control of food and feeds. Furthermore, a new isocratic solvent system has been used for the confirmation and quantification of this mycotoxin by HPLC.

MATERIALS AND METHODS

Microorganisms and Culture Conditions. Micromycetes were obtained from the mycological collection of the Laboratory of Pharmacy, Grenoble [CMPG (Collection Mycology Pharmacy Grenoble)]. They consisted mainly of Deuteromycetes, isolated from different substrates (Seigle-Murandi et al., 1980, 1981; De Hoog et al., 1985; Gams et al., 1990). The three target pathogens, *P. oryzae*, *D. oryzae*, and *G. oryzae* isolated from rice leaves, were supplied by the Department of Phytopathology, International Cooperation Centre in Agronomic Research for Development (CIRAD), Montpellier, France. Stock cultures were maintained on malt extract agar (1.5%) medium (MEA) at 4 °C.

Micromycetes subcultures were grown on MEA medium for a week at 24 °C to provide sufficient inoculum. Mycelia from culture plates were inoculated in 25 mL of liquid malt extract medium in 100-mL flasks. They were grown for a week at the same temperature in a static condition. The mycelia of the fungal strains were filtered off at the end of the growth period, and the culture media were extracted in distilled ethyl acetate (15 mL × 2). The pooled extracts were dried over nitrogen in a water bath at 40 °C. The residues were redissolved in 2 mL of ethyl acetate for UV spectrophotometry and TLC or methanol for HPLC analysis.

***In Vitro* Fungitoxicity Tests.** Tests for antifungal activity of the extracts were done using the disk diffusion method (Devillers et al., 1991). One hundred microliters of the redissolved extracts in ethyl acetate were pipetted onto sterile paper disks (6-mm diameter) and dried (by evaporation) at ambient laboratory temperature. For toxicity tests, 5 mL of the suspension of each of the three test pathogens was separately poured onto MEA medium in Petri dishes (90 mm). The disks impregnated with each extract were placed on the solidified cultures and were incubated at controlled temperatures of 28 °C for *P. oryzae* and

† Laboratoire de Botanique.
‡ Laboratoire de Toxicologie et Ecotoxicologie
§ Laboratoire de Pharmacognosie.

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Table I. ^{13}C NMR of Patulin (1) and Its Acetylated Derivative 2 (δ)

C	1 (CD_3OD)	2 ($CDCl_3$)
2	153.4	146.5[a]
3	147.7	145.7[a]
4	89.8	87.2
6	60.3	60.6
7	109.7	106.7
8	111.0	112.3
9	170.8	168.1
Ac		20.8
		169.1

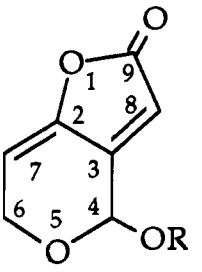
[a] Interchangeable values.

D. oryzae and at 24 °C for *G. oryzae*. These temperatures were earlier found to be favorable for the growth of these phytopathogens (Ou, 1985). Spore germination and growth inhibition diameters (millimeters) were measured after 5 days of incubation. The measured zones of growth inhibition, which were clear areas without mycelia, included the diameter of the paper disks. All experiments were done in duplicate, and the results were arranged in a decreasing order of toxicity based on the level of inhibition of growth of each of the target pathogen. Of the 265 metabolite extracts that prevented the growth of at least one or two of the three pathogens (Okeke et al., 1991), only 67, which were used in the present work (Table III), inhibited the growth of the three rice disease organisms. This work was then centered on these latter extracts. Mycelial growth inhibition activity of pure patulin was also tested at six different concentrations (0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 $\mu g/\mu L$ of ethyl acetate) in cultures of *D. oryzae*, *G. oryzae*, and *P. oryzae*, using the same disk diffusion method.

Mycotoxin Analysis. Preliminary assays were done by measurement of the absorption at UV ranges of both standard commercial patulin (Sigma Chemical Co., St. Louis, MO) as control and extracts in ethyl acetate using an UV–visible recording spectrophotometer (UV-160A, Shimadzu Corp., Kyoto, Japan). This was followed by TLC carried out on silica 60F 254 Kieselgel plates of 0.2-mm thickness (Merck) with (60/30/10 v/v/v) chloroform/ethyl acetate/formic acid (90%) as the development solvent. Patulin was revealed by spraying with a solution of 0.5 g of 3-methyl-2-benzothiazolinone hydrazone (MBTH) hydrochloride (Sigma) in 100 mL of distilled water and drying at 130 °C for 15 min (Scott and Kennedy, 1973). The chromatograms were examined under UV light. Confirmation and quantification of patulin were done by dosage of the standard commercial patulin and the extracts using HPLC. The Shimadzu HPLC equipment consisted of an automatic injector, Model SIL-9A, an UV detector, Model SPD-6A, a pump, Model LC-6A, and an integrator, Model C-R 6A Chromatopac. A 5-μm Kromasil C_{18} column was used (Touzart and Matignon, France). Elution solvents tried were acetonitrile/water/glacial acetic acid (55/45/2 and 45/55/2 v/v/v) (Betina, 1989) and isooctane/methylene chloride/methanol (84/15/1 v/v/v) (Steiman et al., 1989), but the best patulin peak resolution was obtained with methanol/distilled water (10/90 and 15/85 v/v); glacial acetic acid was added in drops until the pH was 3.5. The flow rate was 1 mL/min. Detection limit was 0.01 μg/mL pure patulin at 275 nm. In all cases, patulin in the extracts was identified and quantified by comparison with the standard commercial sample used as both external and internal standards.

Purification of Patulin. Patulin in the extracts was purified by centrifugal thin-layer chromatography (CTLC) performed on silica gel (F-254) with a chromatotron apparatus (Harrison Research). The active compound was eluted from the CTLC plate with the mixture of chloroform/ethyl acetate/methanol (93:5:2, 100 mL).

Confirmation of Patulin. The purified sample was further identified as patulin on the basis of ^1H and ^{13}C NMR data of the natural product and its acetylated derivative (Stothers, 1972) (Tables I and II; Figure 1). NMR spectra of purified samples and pure commercial patulin as standard were carried out with AC 200 Bruker NMR equipment at 200 MHz for ^1H and at 50 MHz for ^{13}C. The solvents used were CD_3OD (δ 3.27 and 49.0) and $CDCl_3$ (δ 7.27 and 77.0).



1 R = H
2 R = Ac

Figure 1. Structure of patulin (1) and its acetylated derivative 2.

Table II. ^1H NMR Spectra of Patulin (1) and Its Acetylated Derivative 2

H	1 (CD_3OD)	2 ($CDCl_3$)
4	5.92 s	6.97 s
6_A	4.33 dd, J = 17.6 and 3.6 Hz	4.51 dd, J = 17.4 and 4.5 Hz
6_B	4.61 dd, J = 17.6 and 2.4 Hz	4.72 ddd, J = 17.5, 2.7, and 1.3 Hz
7	6.00 m	5.96 dt, J = 4.6 and 2.2 Hz
8	6.00 m	6.05 m
Ac		2.14 s

RESULTS AND DISCUSSION

Preliminary spectrophotometric analysis showed that fungal extracts which contained patulin had UV absorption maxima ranging between 272 and 278 nm in ethyl acetate, qualitatively suggesting the presence of this mycotoxin. The standard commercial patulin that was used as control absorbed at 275.2 nm in ethyl acetate and at 275.8 in methanol. These results are close to the UV absorption maxima ranges of 276 nm in ethanol and 278 nm that have been observed after purification of patulin on TLC plates (Engel and Teuber, 1984). In this work, TLC assays of extracts that had UV absorption maxima close to that of the pure patulin revealed the typical yellow spots of the toxin when treated with MBTH. This was also a preliminary assay to determine the presence of the mycotoxin which was further confirmed by HPLC and NMR data (for the high-yielding strains) (Cole and Cox, 1981). The results of the acetylated product are being reported for the first time. Among fungal strains earlier reported as capable of producing patulin were *Penicillium aurantiogriseum*, *Penicillium expansum*, *Penicillium italicum* (Steiman et al., 1989), and *Penicillium melinii* (Betina, 1989). However, high quantities of patulin comparable with those reported by Norstadt and McCalla (1969) were observed in some of the strains under the present conditions. A strain of *P. expansum* produced as much as 2407.3 μg of patulin/mL of liquid malt extract medium (Table III). Another high producer was *Aspergillus giganteus*, which produced 999 μg/mL. The following Deuteromycetes are being documented for the first time as patulin producers: *Alternaria tenuissima*, *Chaetomium atrobrunneum*, *Scopulariopsis flava*, and *Stemphylium* sp. Other fungal strains reported for the first time as patulin producers are *Eupenicillium javanicum*, *Penicillium duclauxii*, and *Penicillium echinulatum*. They produced 911, 937, and 1162 μg/mL, respectively. *Penicillium cyaneum*, *Penicillium diversum*, *Penicillium glabrum*, which produced small amounts of the toxin, and *Penicillium lignorum* with a higher production are, to the best of our knowledge, also being reported for the first time as patulin producers. The minimum quantity of

Table III. Inhibition Diameter toward Three Target Pathogens and Patulin Amount of Extracts from Strains of Micromycetes Cultivated in Liquid Malt Extract Medium

fungi	inhibition diameter (disk diffusion method) toward			patulin	
	P. oryzae	*H. oryzae*	*G. oryzae*	concn, μg/mL of medium	deposit, μg/disk
Ascomycetes					
Chaetomium atrobrunneum	11	10	30	1.5	1.9
Corynascus sepedonium	11	12	26	0.0	0.0
Dichotomomyces cejpii	21	8	14	0.2	0.2
Eupenicillium brefeldianum	26	14	35	0.1	0.1
Eupenicillium javanicum	26	16	20	911.0	1138.7
Eupenicillium sp.	15	18	48	0.9	1.1
Eupenicillium sp.	21	16	15	504.6	630.7
Gymnoascus reessii	26	15	20	0.2	0.2
Myxotrichum chartarum	12	8	14	0.0	0.0
Talaromyces trachyspermus	11	10	31	0.0	0.0
Talaromyces trachyspermus	12	12	27	0.3	0.4
Basidiomycetes					
Chondrostereum purpureum	21	9	8	0.2	0.3
Heterobasidion annosum	17	12	8	0.0	0.0
Coelomycetes					
Coniothyrium sp.	18	11	7	0.7	0.9
Pestalotiopsis sp.	21	15	22	0.7	0.9
Dematiaceae					
Pleiochaeta sp.	21	17	25	0.0	0.0
Alternaria tenuissima	14	10	23	345.4	431.8
Cladorrhinum sp.	9	9	14	0.5	0.6
Drechslera sp.	10	11	14	0.0	0.0
Phialophora hoffmannii	14	20	22	0.2	0.2
Scopulariopsis flava	15	10	24	561.1	701.3
Scytalidium lignicola	22	10	20	0.7	0.9
Stemphylium sp.	16	9	19	303.8	379.7
Ulocladium oudemansii	10	8	11	0.0	0.0
Mucedinaceae					
Acremonium persicinum	11	12	24	0.3	0.3
Acremonium sclerotigenum	19	12	18	0.1	0.2
Aspergillus giganteus	16	14	23	999.1	1248.9
Aspergillus oryzae	26	10	10	0.0	0.0
Aspergillus parasiticus	25	11	12	0.0	0.0
Aspergillus parasiticus	11	15	15	0.0	0.0
Aspergillus petrakii	23	17	10	0.2	0.2
Calcarisporium arbuscula	11	10	12	0.0	0.0
Calcarisporium arbuscula	20	10	13	0.2	0.2
Gliocladium virens	12	8	8	0.0	0.0
Paecilomyces lilacinus	23	13	17	0.3	0.4
Penicillium aurantiogriseum	14	9	8	0.4	0.5
Penicillium aurantiogriseum	11	13	10	0.7	0.8
Penicillium aurantiogriseum	12	8	8	13.8	17.3
Penicillium cyaneum	12	11	24	1.7	2.1
Penicillium cyaneum	22	15	36	0.2	0.3
Penicillium diversum	18	12	12	4.8	6.0
Penicillium duclauxii	16	20	26	936.7	1170.9
Penicillium echinulatum	22	28	30	1162.4	1453.0
Penicillium expansum	14	14	20	314.3	392.8
Penicillium expansum	14	18	18	807.1	1008.8
Penicillium expansum	13	32	30	1121.7	1402.1
Penicillium expansum	24	20	35	2407.3	3009.1
Penicillium funiculosum	10	8	10	0.0	0.0
Penicillium glabrum	15	13	30	1.2	1.6
Penicillium glabrum	15	10	22	1.7	2.1
Penicillium glabrum	11	15	30	2.9	3.7
Penicillium griseofulvum	15	22	25	558.4	698.0
Penicillium hirsutum	17	10	14	1.7	2.1
Penicillium italicum	14	13	32	1.7	2.1
Penicillium italicum	40	26	30	886.7	1108.3
Penicillium lignorum	15	15	18	592.1	740.2
Penicillium melinii	13	20	23	332.6	415.8
Penicillium olsonii	14	9	19	0.0	0.0
Penicillium simplicissimum	10	11	8	0.2	0.3
Penicillium simplicissimum	11	10	10	0.3	0.4
Spicellum roseum	18	12	13.5	0.2	0.3
Trichophyton persicolor	9	8	8	0.1	0.2
Trichothecium roseum	16	13	32	0.8	1.0
Verticillium psalliotae	16	8	8	0.0	0.0
Tuberculariales					
Cylindrocarpon cylindroides	16	11	14	0.2	0.3
Cylindrocarpon ianthothele	16	9	15	0.7	0.9
Cylindrocarpon olidum	15	8	14	0.7	0.9

Table IV. Comparison of the Activity of the Fungal Metabolite Extracts Tested against Three Phytopathogens and of Their Patulin Content

fungal group	number of extracts	number of active extracts	distribution of active extracts with patulin, μg/mL 0	0–10	>10–500	>500
Agonomycetes	4	0				
Ascomycetes	115	11	3	6	0	2
Basidiomycetes	44	2	1	1	0	0
Coelomycetes	64	2	0	2	0	0
Dematiaceae	233	9	3	3	2	1
Mucedinaceae	167	10	3	7	0	0
Aspergillus	74	5	3	1	0	1
Penicillium	163	25	2	12	3	8
Tuberculariales	79	3	0	3	0	0
Yeasts	47	0				
Zygomycetes	70	0				
total	1060	67	15	35	5	12

patulin obtained from the extracts was 0.1 μg/mL and was obtained in extracts from *Eupenicillium brefeldianum*, *Acremonium sclerotigenum*, and *Trichophyton persicolor*. The percentage differences in the production of patulin by the same strains cultured under the present conditions and metabolites extracted in the same manner were also verified and were found to differ by 10–40%, depending on the strain. For example, for *A. giganteus* the quantities of patulin in the medium repeatedly extracted from the same strain grown under the same medium and environmental conditions but in different culture plates differed by 17%, while for *P. expansum* the difference was 26% ($n = 4$). This wide difference in intrastrain and interstrain toxin production, although not earlier quantified, has been observed by other authors (Harwig et al., 1973; Wilson and Nuovo, 1973; Borkowska and Escoula, 1977; Steiman et al., 1989). The phenomenon was recently explained by Devillers et al. (1991), who used a multivariate statistical method to show that the production of antifungal metabolites by various strains of fungi depended on biotic and abiotic factors such as continent of origin, substrates, illumination, and temperature. The strains of fungi that were used in this work were isolated from different substrates and places.

A global comparison of the taxonomic groups of the 67 fungal strains that gave extracts which inhibited the growth of the three target pathogens revealed that 52 of the tested strains produced various quantities of patulin and that *Penicillium* spp. were the dominant patulin producers in terms of both number of strains and quantities of the toxin. For purposes of analysis and due to the often noticed morphological differences from the other members of the Mucedinaceae, *Aspergillus* spp., and *Penicillium* spp. were considered separately (Table IV). Only two strains of Coelomycetes and three of Tuberculariales inhibited the growth of the three target pathogens at the same time, while all five contained small quantities of patulin.

The high quantity of patulin obtained in some of the extracts (Table III) and the fungitoxicity of pure patulin against the growth of the three target pathogens (Table V) indicated that patulin might be responsible for the observed activity of the extracts. However, a correlation analysis of patulin concentrations ≥300 μg/mL in extract and activity of extracts against the individual target pathogens showed very weak correlation coefficients (r^2 = 0.13–0.45) even with the high concentration of patulin in one of the *P. expansum* strains. When correlation was tested without this strain, it remained low for activity against *D. oryzae* and *P. oryzae* while improving slightly for *G. oryzae* ($r^2 = 0.53$), thus showing that the activity cannot be explained by only the levels of patulin in the extracts. Some of the fungi are known to produce other fungitoxic substances (Frisvad and Filtenborg, 1989). These substances may act individually or synergistically with patulin to inhibit mycelial growth of the target pathogens, thus contributing to the observed level of activity of the extracts when compared with that of the pure patulin, especially against *D. oryzae* after 2 days of incubation. On the other hand, the relatively very low levels of patulin in some of the extracts (Table III) show clearly that other active substances, to be identified, are implicated.

Fungitoxicity tests with pure patulin showed that it inhibited the growth of *D. oryzae* and *G. oryzae* at a minimum inhibition concentration (MIC) of 0.50 μg/mL (40 μg/mL of medium) after 2 days of incubation, while at the same time *P. oryzae* was inhibited at MIC of 0.25 μg/mL of medium) (Table V). By the second day of incubation, *D. oryzae* was the least susceptible pathogen, while the other two strains showed almost equal susceptibilities. On the fourth day, *D. oryzae* showed a scanty regrowth of mycelia in the areas of growth inhibition. In these zones the mycelia were decolorized from light brown to white. On the other hand, only a decrease in the diameters of zones of growth inhibition was observed in the cultures of *G. oryzae* and *P. oryzae*. Both conditions may be due to loss of activity of patulin over time or the capability of the pathogens to grow in spite of the toxin or perhaps the pathogens have adapted to use up the toxin. By the fifth day the zones of inhibition remained visible only in the culture plates of *P. oryzae*, representing the longest activity.

In conclusion, therefore, some strains of fungi capable of producing patulin have been identified. This is useful in the quality control of food and feeds. Low correlation was obtained between the antifungal activity of fungal extracts and the quantity of patulin they contained, although the pure toxin was found to be toxic to the target pathogens. Of the 67 fungal strains that showed different levels of activity against the target rice pathogens, 15 did not produce patulin or did not produce enough of the toxin to be detected under the present experimental conditions. Further research is being pursued particularly in the analysis of other secondary metabolites in the extracts.

Table V. Zones of Mycelial Growth Inhibition on Solid Malt Extract Medium of *D. oryzae*, *G. oryzae*, and *P. oryzae* by Pure Patulin, Using the Disk Diffusion Method

patulin concn, mg/L of medium	patulin deposit, μg/disk	diameter, mm, of inhibition zones after incubation for 2–5 days: *D. oryzae* 2 days	3 days	4 days	5 days	*G. oryzae* 2 days	3 days	4 days	5 days	*P. oryzae* 2 days	3 days	4 days	5 days
5	6.25	0	0	0	0	0	0	0	0	0	0	0	0
10	12.50	0	0	0	0	0	0	0	0	0	0	0	0
20	25	0	0	0	0	0	0	0	0	20	12	10	0
40	50	15	8	0	0	24	10	0	0	30	22	17	10
80	100	20	8	0	0	32	20	11	0	30	25	18	10
160	200	25	10	0	0	45	30	21	8	45	35	28	23

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